A NEW BIOPHYSICAL APPROACH TO CORD BLOOD PROCESSING

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Abstract **Umbilical cord blood (CB) is a rich source of stem cells. Stem cells are now being used to treat many diseases. Stem cells are types of mononuclear cells (MNC). Public CB banks must hold thousands of samples which need to be processed and cryo-preserved. Process and storage costs are a significant limiting factor. We have developed and tested a system that can efficiently and inexpensively process CB by utilizing simple components and applying the physical principles of density separation, sedimentation velocity, elutriation and counter flow hydrodynamics. The process is functionally closed and can be performed in a standard laboratory. In addition to standard laboratory equipment including a blood bank centrifuge, the separation system comprises a metering device, bag mounting plates and spring clamps. Using partially lymphocyte depleted human blood as source material we achieved >90% volume reduction with a standardized final volume of 8ml. Median MNC recovery was 73.5% (range 70%-90%) and median total nucleated cell (TNC) recovery was 45%. Total red cells were reduced by >95% and platelets reduced by >90%. This system could offer a truly simple, reproducible and inexpensive CB process which may help to advance CB cell storage and public stem cell use.**

Keywords - **Cord Blood (CB), Cord Blood Banking (CBB), Mononuclear Cells (MNC), Centrifugal cell separation, Elutriation**

I. INTRODUCTION

Cord Blood (CB) is the blood that remains in the umbilical cord and placenta following birth and is routinely discarded with the placenta and umbilical cord. CB is a valid alternative to bone marrow (BM) or mobilized peripheral blood (PB) as a source of hematopoietic stem cells (HSCs) for clinical use [1]. So far, more than 1500 related and unrelated CB transplants have been performed world-wide in malignant and non-malignant diseases [2]. Although clinical results are promising, approximately 80% of CB recipients are represented by pediatric patients [3], [4], [5].

The multipotent-stem-cell-rich blood found in the umbilical cord has proven useful in treating the same types of health problems as those treated using bone marrow stem cells and peripheral blood stem cells (PBSCs). It has been shown in early studies by Broxmeyer et al [6] and others that both term and preterm umbilical cord blood contains a significantly higher number of early and committed progenitor cells when compared with adult peripheral blood. Umbilical CB stem cell transplants are less prone to rejection than either BM or PBSC. This is because the cells have not yet developed the features that can be recognized and attacked by the recipient's immune system. Also, because CB lacks well-developed immune cells, there is less chance that the transplanted cells will attack the recipient's body, a problem called graft versus host disease (GVHD). Both the versatility and availability of umbilical CB stem cells makes them a potent resource for transplant therapies.

Cord blood banking (CBB) is cryopreserving CB for future use. There are two CBB options. The first is Family Banking where Parents may choose to save their child's CB for use by their child or another family member in the event a life-threatening disease develops. The second option is Public donation where parents choose to donate their child's CB to the community, similar in principle to a routine blood donation. Under these circumstances, the CB would be released for use by any qualified recipient, or perhaps for scientific research.

The large scale processing of CB of volumes typically between 40ml and 150ml is increasingly becoming important in order to establish sufficiently large public cell banks. A bank large enough to serve a country the size of Egypt would need to contain more than 50,000 samples. Minimizing the final cell volume is very important to reduce long term cryogenic storage costs.

Processing can be done manually but requires a pharmaceutical quality cleanroom with labor intensive procedures. Semi-automated processes are available with devices such as the GE Healthcare AXP [7], the Biosafe Sepax [8] and experimentally the Gambro Cobe Spectra [9]. Manual processes such as the Rubenstien [10] process can be regulated relatively easily but only when performed within a strictly monitored good manufacturing practice (GMP) cleanroom. Other new manual systems rely on a filter technique [11] which captures white cells from the CB passing through which can be subsequently washed off and concentrated. Medical devices that automate any part of the process are subject to strict regulatory process monitoring and reporting as described by the good automated manufacturing practice regulations (GAMP). Most current manual processes tend to have low consumable costs with the possible exception of filters but high staffing and facilities costs. More automated processes have relatively high consumables and device costs but with relatively low facilities costs where a cleanroom is not considered essential.

II. BIOPHYSICAL BACKGROUND

A. Principles of centrifugal cell separation

Human blood cells have a range of densities between 1.03 and 1.14 g/cm^3. CB has the whole range of cells as in normal human blood and the constituent stem cells occupy the range between approximately 1.04 to 1.08 g/cm3. The stem cells in effect occupy the same region as mononuclear cells (MNC) as indicated by Monocytes and Lymphocytes in Fig. 1.

When spun in a centrifuge at a force measured as a multiple of gravity (g), cells separate based on relative density. The key of Fig. 1 would therefore indicate the 'stacking' of cells in order with the least dense (platelets) at

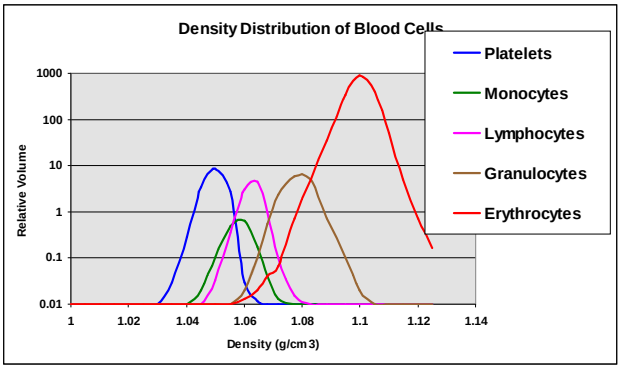

Fig. 1.Density distribution of blood cells.

the top of the centrifugal field and the most dense (erythrocytes or red blood cells, RBC) at the bottom

Blood cells are suspended in plasma, a fluid having a density > 1.00g/cm^3 but normally lower than 1.03g/cm^3. In a centrifugal field cells travel through plasma and eventually assume their particular relative density position. The rate of travel or sedimentation velocity for each cell type is dependent on its density and size, and suspension fluid viscosity.

Sedimentation velocity (S_v) is defined by Stoke's Equation

$$S_v = (\omega^2 R)\, d^2\, (\rho-\rho_0) / 18\mu \qquad (1)$$

Where ω = centrifuge speed, R = rotation radius, d = cell diameter, ρ = cell density, ρ_0= suspension media density, μ = reciprocal of suspension media viscosity.

However cells spun at a high enough g x time will allow equilibrium of cell density based positions to be reached and therefore S_v becomes irrelevant. Incomplete separation will leave certain cells at various points in their travel. After equilibrium is reached further centrifugation will not change cell relative positions but will pack them more densely displacing plasma.

All centrifugal separation devices use this technique to sort cells but differ in the way they collect them. A standard centrifuge will separate cells in a bag or tube which is carefully removed and cells extracted by mechanical pressing into other bags or extracted by pipette. The principle of collecting cells in this way is well proven but the application and control of cells exiting the bag is crude and processes often inconsistent. The typical target cells are MNC which layer on top of the granulocytes. This position is often referred to as the 'interface' and the MNC and granulocytes collectively referred to as the buffy coat (BC).

The Thermogenesis/GE Healthcare AXP device uses a flow control device within a standard centrifuge. Cells are centrifugally separated within the system but whilst spinning are sorted into separate components. Currently it achieves a collection recovery of >90% of components within the density range of 1.03 to 1.10g/cm^3 in a volume of 20ml from a 100ml blood sample.

The Biosafe Sepax device has an integrated centrifuge and separation device which in effect is a vertical axially spinning cylinder. The RBC will occupy the outer edge of the cylinder with the other cells stacking within. A piston at the bottom of the cylinder when pushed upwards expresses cells in density order lowest first from the inside of the cylinder outwards. In this way cells can be collected externally separately. The Sepax device can collect the same density range as the AXP in a volume down to 8ml but with an MNC collection recovery of 65-70%.

The Cobe/Gambro Spectra device, also an integrated device, uses a ring channel within the centrifugal field and relies on separation dynamics to separate and concentrate cell types and by enriching the high density layer lifts the cells and presents them to a collection tube where they are drawn out and collected. Experimental results [12] show a collection recovery of MNC in a similar range to the Sepax device with a volume of about 5ml.

Each of these three devices separate and collect cells during centrifugal separation. A standard centrifuge separates cells but the collection is done remotely outside of the centrifugal field. Once fully separated 100ml of cord blood will have a total volume of mononuclear cells of <1ml but to collect this efficiently, due to layer concentration and stability, a much greater volume is collected. However low volumes in the range of 5-10ml are desirable but 20ml acceptable for cord blood processing. Each device also allows cells to be handled in a fully environmentally closed system in consistently reproducible procedures.

The three proprietary systems as described use relatively high cost disposables (approximately US$120). Large scale use might therefore be prohibitively expensive.

Another cell separation system produced by Gambro BCT is the Elutra [13] which uses elutriation to sort cells both by density and size.

B. Principles of Elutriation

Elutriation is a similar but opposite effect to Sedimentation. Elutriation is defined as "The process of separating the lighter particles of a powder from the heavier ones by means of an upward directed stream of fluid (gas or liquid)" [14]. One of the oldest examples of this is the separation of wheat, the grain from chaff where the wheat husks are shaken in a sieve and moved to either create a draft from beneath the sieve or across the top to selectively remove the lighter chaff, it being carried away by the flow of air, leaving the grain in the sieve.

Elutriation in our setting is where the flow of fluid out of the bag is high enough for the cells to overcome the 1g force holding the interface stable. The effect of this will be to selectively lift cells from the interface based upon density and size, a converse effect to sedimentation. Referring to Fig. 1 and Fig. 2 where the platelets, the smallest and lightest cells, would be eluted first. In true elutriation cell size has a greater contribution than density so the next cells eluted would tend to be lymphocytes and RBC.

C. Elutriation may be desirable or undesirable

The purity and recovery of desired cells are the most important factors to consider. Referring to Gambro Elutra data for a given cell surface cross sectional area the first cells eluted will be platelets, then lymphocytes and red cells, and

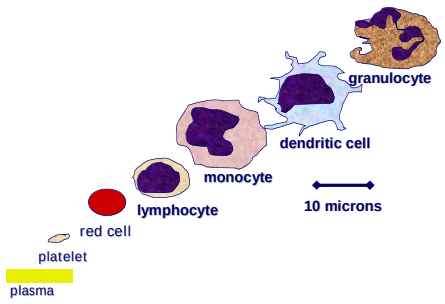

Fig. 2.Blood cells sizes

then monocytes. Our system is not quite the same as Elutra which uses counter flow hydrodynamics, where within a centrifugal field there is a counter flow of media in the opposite (centripetal) direction which creates a viscous drag force according to the equation:

$$V_{CHV} = F / O(r') \quad (2)$$

Where: F = flow rate of elutriation media, O(r') = cross sectional area of the sedimentation chamber as a function of r', the radial distance from the centre line of the centrifugal force.

Elutra also uses fluidized particle beds of each cell type which due to the balance of centrifugal and centripetal forces as described previously each cell type can be held in suspension.

In our system the force acting is only 1 x g. A minimal fluid flow will occur through the cells but only as the cell mass is compressed displacing plasma rather than by one externally supplied and controlled.

Our target cells are MNC and we want to minimize platelet and RBCs contamination if possible. Platelet contamination is reduced due to the even distribution in plasma and low concentration as previously described. True elutriation would predict lymphocytes, RBCs and monocytes to be consecutively eluted. However as the RBCs are the most dense cells and not held in a fluidized bed we would predict that their momentum may well hold back most RBC allowing lymphocytes and monocytes to flow out preferentially. If this hypothesis is true then we will have a high recovery and highly purified MNC product.

We also want the final MNC product to be in a low volume of about 5-8ml. The MNC will be cryopreserved and minimized volumes are important to reduce the required amount of cryo-protectant dimethylsulphoxide (DMSO) and storage volume.

If elutriation is desirable the narrowing bag neck is important as the cell surface area determines the cell type eluted for any particular fluid outflow rate. An empirical study will determine the flow rates and associated cell types. If elutriation is undesirable then the outflow rate must be kept below that required to cause elutriation of desired cells leaving them in their appropriate density separation position.

The question remains: Is it possible to use low cost standard centrifugal separation and manual extraction to achieve a procedure offering consistent high cell recoveries and low volume in a closed system?

Based upon the understanding of the proprietary systems and current manual, closed system extraction techniques, we have devised a simple process to achieve this. In practical terms we must have a process that maintains interface stability post centrifugation and prior to cell expression and one that controls fluid flow during cell expression to control elutriation of the desired cells.

III. Processing System

We have developed and tested a manual process that can be operated without a cleanroom using standard laboratory equipment with the addition of an inexpensive non-calibrated metering device, centrifugal adaptor/press plates and spring clamps. This system, although simple in its final application, has been devised and developed using the application of the physical principles of density separation, sedimentation velocity, elutriation and counter flow hydrodynamics.

Our separation system comprises 2 main elements:

1) An assembly with transparent rigid plates into which the cell bag is placed and centrifuged. This performs several functions. It holds the bag in a standardized position and forms it into a standardized shape. It allows for easier removal of the bag and forms part of the press mechanism for cell expression. Transparency allows for easy optical expression observance.

2) A peristaltic metering device for precise variable control of fluid outflow.

IV. METHODOLOGY

We ran several procedures and changed only the rates of fluid expression from the bag. Below we describe the most significant three.

The objective was to establish the best approach on which to base the final protocol from which results can be taken and conclusions drawn.

The starting cell product of nominal 100ml is collected into a bag and spun in a standard centrifuge at 450g for 15min. This is enough force to fully separate cells of density >1.04g/cm3 but cells of density below this i.e. platelets have such a slow sedimentation velocity that they remain in fairly even concentration throughout the plasma. This is an often

Fig. 3 Blood collection bag

used procedure in blood banks when what is known as "platelet rich plasma" is required.

The separation cell bag (Fig. 3) used has a gradually narrowing neck which gives a greater visible depth of cells as plasma is expressed and the cells at the interface rise. At a particular flow rate for each cell type as the cells enter the reducing cross sectional area of the bag, elutriation will take place.

The cell bag and plates after removal from the centrifuge was mounted and a spring compressor externally attached. The bag exit tubing was loaded into the peristaltic metering device which was set to allow certain exiting flow rates.

Consecutive 10ml volume aliquots of expressed fluid were collected and analyzed as in tables I, II and III below where:

WBC = total white blood cells ($x10^9$/L) (=TNC)
LYM = lymphocytes ($x10^9$/L)
MXD = monocytes + other mononuclear cells ($x10^9$/L)
NEUT = neutrophils ($x10^9$/L)
Hct = Haematocrit = % red blood cells (rbc)
Plt = Platelet count ($x10^9$/L)

The first observation to make is that platelet S_v had not been reached as shown by fairly constant counts in most aliquots. The significant individual aliquots are number 7 at 5.75ml/min (Table 1.) and number 6 at 28.8ml/min (Table 2.) The actual aliquot number where cells are seen is not significant as process variances such as actual cell volumes will cause this variability and as aliquots are chosen manually by sight, cells can be selected appropriately.

These results seem to suggest that elutriation has begun or increased with the increased flow rate as both WBC and lymphocyte counts have doubled. The significant increase

TABLE I
EXPRESS RATE SET TO 5.75ml/min

id	Vol	WBC	LYM	MXD	NEUT	Hct	Plt
PRE	102ml	1.7	0.2	0.1	1.4	27.4	34
1	0-10ml	0	0	0	0	0	26
2	10-20ml	0	0	0	0	0	27
3	20-30ml	0	0	0	0	0	30
4	30-40ml	0	0	0	0	0	31
5	40-50ml	0	0	0	0	0	32
6	50-60ml	0	0	0	0	0	31
7	60-70ml	3.8	0.3	0.3	3.2	13.5	73
8	70-80ml	5.6	0.4	0	0	59.3	22
9	80-90ml	3.2	0.5	0.2	2.5	63.5	17

TABLE II
EXPRESS RATE SET TO 28.8ml/min

id	Vol	WBC	LYM	MXD	NEUT	Hct	Plt
PRE	100ml	1.7	0.2	0.1	1.4	27.4	34
1	0-10ml	0	0	0	0	0	21
2	10-20ml	0	0	0	0	0	20
3	20-30ml	0	0	0	0	0	18
4	30-40ml	0	0	0	0	0	17
5	40-50ml	0	0	0	0	0	23
6	50-60ml	8.4	0.6	0.8	7.0	24.6	94
7	60-70ml	3.6	0.5	0.3	2.8	60.8	22
8	70-80ml	1.3	0.5	0.2	0.6	65.2	8
9	80-90ml	2.0	0.5	0	1.5	70.9	18

TABLE III
EXPRESS RATE SET TO 28.8ml/min and reduce d to 5.75ml/min to harvest

id	Vol	WBC	LYM	MXD	NEUT	Hct	Plt
PRE	100ml	1.7	0.2	0.1	1.4	27.4	34
1	0-10ml	0	0	0	0	0	25
2	10-20ml	0	0	0	0	0	20
3	20-30ml	0	0	0	0	0	22
4	30-40ml	0	0	0	0	0	17
5	40-50ml	0	0	0	0	0	23
6	50-60ml	7.65	1.5	0.8	5.35	15.4	90
7	60-70ml	5.6	0.3	0.3	5.0	30.8	20
8	70-80ml	3.3	0.1	0.2	3.1	55.2	11
9	80-90ml	2.0	0.1	0	1.8	70.9	8

TABLE IV
Final protocol results

id	Pre Vol	WBC	LYM	MXD	Hct	Plt
PRE		2.6	0.4	0.4	26.7	56
1	118ml	16.0	4.1	4.4	12.1	101
2	90ml	15.8	4.1	3.9	14.6	96
3	96ml	14.2	3.6	4.2	18.7	88
4	105ml	15.2	3.8	4.8	16.0	115

in Hct could be due to eluting of the RBC or the cutting down further into this layer. Of most concern is the loss of MNC which appear to be evenly distributed within the RBC which cannot be explained by density separation alone.

We postulated that a combination of different flow rates might increase MNC recovery and RBC depletion. After several attempts and in an effort to keep the process simple we arrived at the flow settings of 28.8ml/min until the cells entered the narrowing neck region of the bag and then changed to 5.75ml/min.

Table III shows this hybrid approach in an attempt to optimize target cell recovery, maintain the minimal platelet contamination and reduce the excessive RBC contamination.

It would appear from the data that this has the effect of lifting the lymphocytes whilst the RBC seems to drop back into their relative density position.

We therefore based our final protocol on this hybrid approach. We began the collection just as the MNC layer was observed and targeted a final volume of 8.0ml.

V. RESULTS

We performed 4 procedures with another batch of partially lymphocyte depleted blood with results as shown in Table IV. The MNC recoveries ranged from 70% to 90% with a median of 73.5%, and WBC or TNC recoveries ranged from 42% to 54% with a median of 45%. Total red cells were reduced by > 95% and platelet depletion was >90%.

VI. DISCUSSION

The objective of this study was to test the hypotheses related to the various physical separation and flow characteristics of cells within our system. The overall aim however was to produce a practical and realistic, rather than academic protocol.

With an n=4 statistical relevance cannot be drawn from these results although we consider the consistency of final results good.

The consistency of the procedures prior to the final set was poor. We feel that this was related to the very different cell counts in the starting blood product and the varying loading positions within the centrifuge. Also the handling of the bag post centrifugation and positioning of spring clamps on the plates for cell expression may well have contributed to this.

We actually found that slight cell interface disturbance post centrifuge actually improved MNC yield.

There are many issues that still need to be addressed. Low cell counts may give rise to counting errors and actual CB may have up to 10 times the WBC and MNC cell numbers of the blood product that we used. Also cell densities are of a range as in fig 1. and the starting blood product may have already been depleted of a particular density range of cells and skewed the results.

Thus the partially lymphocyte depleted blood product used is somewhat unrepresentative of typical cords and may yield significantly different results.

Traditional CB processors measure several criteria to establish procedural success. One of these is WBC or TNC recovery which should be >75%. With our 45% recovery we fall significantly below this. Most of our WBC losses are of granulocytes which in our opinion add nothing to the stem cell product as these cells die during cryopreservation. This is still a controversial and debated point.

We have not mentioned or discussed other elements of CBB such other consumables and equipment, and the stages of cell cryopreservation, as we consider these broadly common to all techniques.

This system and protocol are soon to be used to process real CB, at which point many of the questions will be answered, and a judgment made as to its applicability.

V. Conclusion

We do not feel that we can scientifically explain the interaction between sedimentation velocity and density and elutriation cell separation in our system, but hope that our study results can nevertheless be practically useful.

If successfully trialed and validated on real CB this inexpensive, relatively low technology process can provide large scale rapid CB processing. In a large blood bank centrifuge 12 cords can be spun at one time with each CB only requiring another 15min to process. We estimate therefore that with planned workflow and parallel processing, single system components except for 2 metering devices and scales, 2 operators can process up to 100 cords per day without a cleanroom.

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